JOURNAL OF CLINICAL MICROBIOLOGY, July 1996, p. 1637–1640 Vol. 34, No. 7
0095-1137/96/$04.00+0

PCR of Peripheral Blood for Diagnosis of Meningococcal Disease

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Received 4 December 1995/Returned for modification 30 January 1996/Accepted 1 April 1996

Meningococcal disease is normally suspected on clinical grounds and is confirmed by isolation of *Neisseria meningitidis* bacteria from blood or cerebrospinal fluid or, more recently, by serology or PCR of cerebrospinal fluid. Achieving confirmation of a clinical diagnosis of meningococcal disease has become more difficult in the last few years. The prehospitalization administration of parenteral benzylpenicillin normally renders blood cultures sterile, and lumbar puncture is undertaken less frequently, especially in young children. We evaluated PCR for the detection of meningococcal DNA in 80 blood samples taken from patients with known or suspected meningococcal disease or from patients with other diagnoses (negative controls). Both the sensitivity and the specificity of the test were 100% for patients with confirmed cases of meningococcal disease when the blood buffy coat was used (83 to 100% sensitivity and 87 to 100% specificity with 95% confidence limits). Positive PCR results could be obtained from both blood buffy coat and serum samples. Sensitivity was unaffected by prior antibiotic treatment. PCR is a rapid, sensitive test that may be used to confirm a diagnosis of meningococcal disease by using peripheral blood samples. Introduction of this test into clinical laboratories may in some cases obviate the need for lumbar puncture to be performed on patients with suspected meningococcal disease. Our results demonstrate that a substantial number of cases of meningococcal disease are not confirmed by conventional techniques and remain undiagnosed. If the PCR test described here was widely applied, the number of cases of meningococcal disease ascertained might rise by as much as 60% greater than that recognized at present. It is likely that we are in a prevaccination era for meningococcal disease. Better case ascertainment is urgently required to assess the need for vaccines, to determine their costs and benefits, and to monitor their efficacies.

Meningococcal disease remains an important health problem in the United Kingdom and worldwide. Conjugated polysaccharide vaccines for both serogroup A and serogroup C strains of *Neisseria meningitidis* are currently available (1) and are undergoing clinical trials. However, in the United Kingdom most cases of meningococcal disease are caused by serogroup B strains, and the development of a serogroup B conjugated vaccine has been hampered by the poor immunogenicity of the serogroup B polysaccharide. Multivalent serogroup B vaccines based on outer membrane proteins (2, 8, 11, 12) are being evaluated. Several other candidate serogroup B vaccines are also under development. It is therefore likely that we are in a prevaccination era for meningococcal disease in the United Kingdom. Accurate case ascertainment is vital in order to evaluate the burden of disease and then to monitor the effects of introducing new vaccines.

Confirmation of the diagnosis of meningococcal disease is becoming increasingly difficult. Meningococcal disease is often suspected first on clinical grounds and is then confirmed by the isolation of *N. meningitidis* from blood or cerebrospinal fluid (CSF) or by microscopic detection of gram-positive diplococci in CSF. Confirmation of the diagnosis can be made from CSF in almost 90% of patients (3). However, lumbar puncture is sometimes contraindicated because of raised intracranial pressure (10) and may be unsuccessful in obtaining a sample of CSF because of technical difficulties. There is a trend away from the use of lumbar puncture (4a), especially in young children and particularly if a clinical diagnosis of meningococcal disease seems clear-cut.

Meningococci can be recovered from cultures of blood from about 50% of untreated patients with meningococcal disease. However, preadmission antibiotic treatment, which reduces mortality and is recommended by the United Kingdom's Chief Medical Officer, reduces the chance of a positive blood culture to 5% or less (5).

PCR has been used extensively to amplify and detect DNA from clinical samples. We demonstrated previously the value of a PCR designed to amplify a segment of the meningococcal insertion sequence IS*1106* (6) for the specific and sensitive detection of *N. meningitidis* DNA in clinical CSF specimens (9). The test had both a sensitivity and a specificity of greater than 90%. The test is now used routinely by Meningococcal Reference Laboratories in the United Kingdom to detect meningococcal DNA in CSF samples from patients who are culture negative because of prior antibiotic treatment. However, a PCR test has not so far been applied to the detection of meningococcal DNA in specimens of peripheral blood. We demonstrate here the value of such a test. Our technique proved to be sensitive and specific and confirmed the diagnosis of meningococcal disease in a substantial number of patients for whom no other confirmatory laboratory method was available.

MATERIALS AND METHODS

Patients and specimens. Peripheral venous blood samples (for standard hematological tests and anticoagulated with EDTA) were those taken at the time of hospital admission from patients with suspected meningococcal disease and from controls. The clinical features of the patients and the specimens examined in the study are as follows: patient category 1, proven meningococcal septicemia and positive blood culture; patient category 2, confirmed meningococcal disease

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TABLE 1. Primer and probe sequences

Primer or probe	Primer sequence 5′-3′	Nucleotides within IS*1106*
1	ATTATTCAGACCGCCGGCAG	850–869
8	TGCCGTCCTGCAACTGATGT	1161–1141
Capture probe	Biotin-GTACCGATGCGGAAGGCTAT-	1001–1020

by positive CSF culture or microscopy but blood culture negative or blood not taken; patient category 3, suspected meningococcal disease (fever with hemorrhagic rash with or without meningism) but no organisms isolated; patient category 4, possible meningococcal disease (polymorphs in CSF, no organisms isolated or seen, and no rash); patient category 5, no suspicion of meningococcal disease (see below for details of clinical diagnoses); and patient category 6, known (unblinded) negative controls. Control specimens (patient category 5) included patients with viral meningitis ($n = 3$), pneumococcal meningitis ($n = 3$), *Haemophilus* meningitis ($n = 2$), tonsillitis ($n = 1$), otitis media ($n = 1$), rickettsialpox ($n = 1$), vaccine reaction ($n = 1$), and thrombocytopenia ($n = 2$). All of these controls had either a hemorrhagic rash or possible symptoms of meningeal irritation. Control patient category 6 included routine blood samples taken from patients at the time of hospital admission for whom there was no suspicion of meningococcal disease. These latter samples were handled unblinded and were used to establish the cutoff for the test. Samples were collected from patients in Gloucester, Hereford, Plymouth, and Cheltenham, United Kingdom. Almost always after overnight storage at 4°C, the samples were centrifuged and divided into buffy coat, serum, and erythrocyte fractions. The samples were coded, stored at −20°C, and transported to the University of Surrey for DNA extraction and PCR analysis.

Positive control. In order to measure the sensitivity of the PCR, a serum sample was spiked with a suspension of *N. meningitidis* cells at a known concentration. The control *N. meningitidis* strain used in the study was clinical isolate L92/337, a W135 nontypeable P1.6 strain obtained from the Manchester Public Health Laboratory Service. The culture was grown on GC agar base (Oxoid) containing 5% hemoglobin (Becton Dickinson), yeast autolysate, Vitox growth supplement (Oxoid), and VCNT antibiotic supplements (containing vancomycin, colistin methane sulfonate, nystatin, and trimethoprim) (Oxoid). One colony from a fresh plate culture was resuspended in 1 ml of phosphate-buffered saline from which serial dilutions were made. Direct plating of this suspension showed the cell concentration to be 3×10^6 to 4.5×10^6 CFU/ml. Whole blood was obtained from a healthy volunteer via peripheral venipuncture. Aliquots of the serum fraction of 80 μl were inoculated with 20 μl of the diluted bacterial suspensions.

PCR sample preparation. DNA was purified from 50 μl of blood buffy coat or serum sample by the method of Boom et al. (4). The sample was lysed in 900 μl of lysis buffer (120 g of guanidinium thiocyanate dissolved in 100 ml of Tris-HCl [pH 6.4], 22 ml of EDTA [pH 8], 2.6 g of Triton X-100) in the presence of 40 μl of a diatom (Celite; Aldrich Chemicals) suspension. After 10 min of incubation at room temperature, the diatoms were pelleted; this was followed by washing in 1 ml of wash buffer (120 g of guanidinium thiocyanate dissolved in 100 ml of Tris-HCl [pH 6.4]), ethanol, and acetone. The pellet was dried for 10 min at 56°C before the DNA was eluted from the diatoms by heating at 56°C for 10 min in 30 μl of Tris-EDTA (TE). The DNA samples were stored at −20°C prior to PCR.

PCR amplification. The samples were handled in batches of 10, with two extraction blanks. A total of 3 μl of diatom eluent was used in a 25-μl PCR mixture comprising 1.25 U of *Taq* polymerase, PCR buffer, 8% glycerol, 150 ng of primers (Table 1), 200 μM (each) dGTP, dCTP, and dATP, 240 μM dUTP, 0.25 nmol of digoxigenin-dUTP (DIG-dUTP), and 0.25 U of uracil glycosylase (Boehringer Mannheim). The PCR conditions were 1 cycle at 37°C for 10 min and 95°C for 10 min; 32 cycles at 95°C for 25 s, 61°C for 40 s, and 72°C for 1 min; and then holding at 72°C. The PCR products were subsequently stored at −20°C. For each set of PCRs a positive (*N. meningitidis* DNA) and negative (water) control were included.

Detection of PCR products by DIG-PCR ELISA. The DIG-PCR enzyme-linked immunosorbent assay (ELISA) was available as a kit and was performed essentially as described by the manufacturer (Boehringer Mannheim). DNA from 20 μl of the PCR mixture was denatured; this was followed by hybridization to a biotinylated capture probe. The capture probe was immobilized onto the surface of a streptavidin-coated ELISA plate, and bound hybrid was detected with an anti-DIG peroxidase conjugate and the colorimetric substrate ABTS [2,2′-azino-bis(3-ethylbenzthia-zoline-6-sulsonic acid)], according to the manufacturer's instructions. An ELISA plate reader was used to record the result. The biotinylated probe was synthesized and purified by Oswel (Edinburgh, Scotland). The primers for the PCR and the capture probe sequence were based on the meningococcus-specific insertion sequence IS*1106* (6). The primers were designed to generate a 331-bp fragment of IS*1106* from *N. meningitidis*. The PCR capture probe sequence was internal to the PCR product (Table 1).

RESULTS

Sensitivity and specificity of PCR amplification. The PCR that we have described previously for the detection of *N. meningitidis* DNA targets the meningococcus-specific insertion sequence IS*1106* and was shown to be both sensitive and specific (9). However, preliminary experiments established that this PCR test had insufficient sensitivity to detect meningococcal DNA in clinical blood samples. A feature of the previous PCR test was that different strains of *N. meningitidis* sometimes gave a main product band of 596 bp plus additional bands of various sizes in different strains, effectively diluting the PCR signal. This was presumably due to variation in the organization of the multiple copies of IS*1106* in these strains. To increase the sensitivity we sequenced some of the variant PCR products (data not shown). The sequence data for the variant bands indicated that illegitimate recombination events within IS*1106* were probably responsible for these extra bands. For instance, a smaller PCR product of 475 bp was sequenced and was shown to be due to a deletion of 121 bp from the sequence of IS*1106*, between bases 1188 and 1308. The ends of the deletion were flanked by a short direct repeat of the 5-bp sequence AGCCT. We therefore designed new primers to amplify a 331-bp common sequence for all of the variant products. A further modification was the incorporation of deoxyuridine into the PCR products so that PCR product contamination could be prevented with uracil glycosylase (7).

Although the use of the new primers increased the sensitivity of the PCR test 5- to 10-fold, this was still not sufficient for the detection of DNA in many test blood specimens. Although we could improve the sensitivity further by, for example, Southern blotting of PCR products and detection by DNA hybridization with radiolabelled DNA probes, we felt that this technology was not compatible with use in a clinical diagnostic laboratory. Therefore, to increase the sensitivity further, we used the DIG-PCR ELISA system for the sensitive detection of the PCR product. In this system, DIG is incorporated into the PCR product. The product is then captured onto a streptavidin-coated microtiter well plate by DNA hybridization with a biotinylated capture probe. The captured PCR product is then detected with an enzyme-conjugated antibody against DIG and a chromogenic substrate and is quantitated with an ELISA plate reader.

The sensitivity of the PCR was determined by using 10-fold dilutions of the *N. meningitidis* genomic DNA and then detection by DIG-PCR ELISA. The ELISA could detect 2 fg of genomic DNA (equivalent to approximately one genome). The ELISA system was shown to be 10 to 100 times more sensitive than electrophoresis through an agarose gel and then ethidium bromide staining of PCR products. The specificities of the primers were tested by PCR amplification of DNA from the following bacterial species: *Streptococcus pneumoniae*, *Streptococcus faecalis*, *Haemophillus influenzae*, *Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Mycobacterium bovis* BCG, *Neisseria gonorrhoeae*, and *Neisseria lactamica*. Only *N. meningitidis* tested positive (data not shown).

We next determined the sensitivity of the assay with spiked serum. Aliquots of serum taken from a healthy individual were inoculated with a dilution series of a suspension of *N. meningitidis* cells. DNA was extracted and amplified by PCR, the PCR product was detected by the DIG-PCR ELISA. The maximum sensitivity of the assay was 30 to 45 CFU/ml of serum.

Detection of *N. meningitidis* isolates in clinical specimens. Preliminary experiments were performed with known negative control specimens (patient category 6) and samples spiked with meningococcal DNA, and a cutoff value of greater than 0.5 absorbance units indicating a positive result for the detection of meningococcal DNA was established. Coded clinical specimens from patients with confirmed and suspected meningo-

TABLE 2. DIG-PCR results for blood buffy coat and serum specimens

Patient category[a]	Buffy coat			Serum		
	No. of specimens	No. of specimens PCR positive	Sensitivity (%)	No. of specimens	No. of specimens PCR positive	Sensitivity (%)
1	10	10	100	9	8	89
2	15	15	100	17	14	82
3	10	10		10	8	
4	18	5		19	1	
5	14	0		14	0	
6	12	0		12	0	

[a] See text for the clinical features of the patients and the specimens examined.

coccal disease and controls were then examined by the DIG-PCR ELISA. The number of specimens are given in Table 2, and the clinical criteria used to classify the patients are given in Materials and Methods. Controls (patient category 5) included specimens taken from patients with confirmed disease and with clinical manifestations similar to those of meningococcal disease, including bacterial meningitis other than meningococcal meningitis (three pneumococcal and two *Hemophilus* specimens), viral meningitis (three specimens), and rickettsialpox (one specimen).

After performing the test on all of the specimens, the code was broken and the results are presented in Fig. 1 and Table 2. The sensitivity of the test was determined by using specimens only from patients in categories 1 and 2. These were patients with confirmed meningococcal disease as determined either by culture or by the presence of gram-negative intracellular diplococci in CSF. The sensitivity of the assay for these groups combined was 89% for serum (95% confidence limits, 65 to 96%) and 100% for blood buffy coat (95% confidence limits, 86 to 100%). The specificity of the test was determined by using specimens from patients (category 5) with confirmed diagnoses of diseases other than meningococcal disease. The sensitivity was found to be 100% for both serum and blood buffy coat (95% confidence limits, 87 to 100%). It should be emphasized that although the empirical sensitivity and specificity of the test in our study were both 100%, the actual sensitivity and specificity may be significantly lower (83 to 100% and 87 to 100%, respectively, with 95% confidence limits) because of sample size limitations.

Ten buffy coat specimens were obtained from patients for whom there was a strong suspicion of meningococcal disease (fever with hemorrhagic rash) but from whom no organisms were isolated (Table 2, patient category 3). By using the PCR test with the blood buffy coat, confirmation of meningococcal infection was obtained for all of these patients. Four of the 25 patients with confirmed invasive meningococcal disease and 7 of the 10 patients with suspected meningococcal disease had been treated with parenteral benzylpenicillin before the specimen was collected. Blood cultures were uniformly negative for these patients, but they all gave a positive PCR result. Buffy coat specimens from a further 18 patients with evidence of bacterial meningitis but without a hemorrhagic rash (Table 3, patient category 4) were examined, and 5 of these were found to be positive for meningococcal DNA. None of these patients had been treated with antibiotics.

DISCUSSION

This is the first report of a controlled, blinded trial that evaluated PCR for the detection of *N. meningitidis* in blood. The PCR-DIG ELISA had an excellent sensitivity and specificity. The test is as rapid (results are available in less than 8 h) as conventional culture (results are available in 12 to 36 h). Unlike culture, the test does not seem to be affected by prior antibiotic therapy. We do not have accurate data on the time between antibiotic administration and sample collection, although it is likely to between 1 and 4 h for most patients. Data were available for a single antibiotic-treated patient from whom a blood sample was collected at the time of hospital admission and from whom a second sample was taken 2 days later. The first specimen was positive when the PCR was performed with both buffy coat blood and serum, whereas the second specimen was positive only with the buffy coat blood. However, further studies will have to be performed to determine the time period after treatment that DNA may remain detectable by PCR. Serology can be used to demonstrate evidence of meningococcal infection; however, serology depends on the generation of an antibody response and is therefore not helpful in immediate patient management. Latex agglutination tests are available for the detection of antigen, but only in CSF specimens. The PCR test may be performed immediately on peripheral blood samples obtained at the time of admission from patients with acute cases of disease. Although the blood buffy coat gave the highest sensitivity, serum specimens from most of these patients were also PCR positive, indicating that meningococcal DNA was present in our serum specimens. It is possible that DNA may be transiently present in the serum of patients with meningococcal disease. Alternatively, meningococcal DNA may have been released from lysed cells into the serum fraction during sample preparation. Blood samples were collected into tubes containing EDTA, which would inhibit any DNase that was present. Our PCR blood test is practical and convenient and could be readily applied in clinical laboratories. PCR of blood offers the additional advantage that at least one peripheral blood sample for routine hematological analysis is collected on admission from virtually all patients with suspected meningococcal disease. Sample collection therefore confers no additional hazard to or inconvenience on the patient. The test described here may therefore be of particular benefit for the diagnosis of meningococcal disease in patients in whom lumbar puncture is contraindicated or in whom antibiotic treatment before specimens were taken has rendered blood or CSF sterile.

Many clinically diagnosed cases of meningococcal disease are not confirmed by blood culture because of the increasing

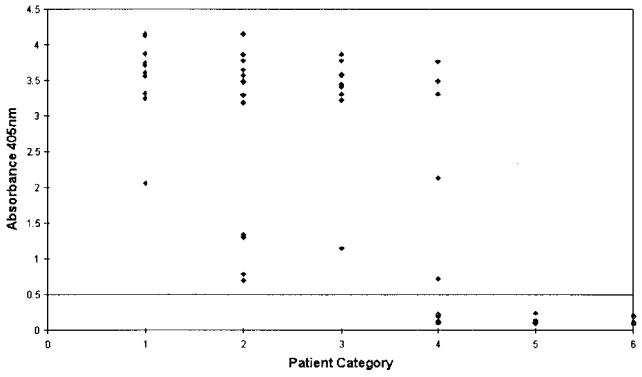

FIG. 1. Results of DIG-PCR ELISA detection of meningococcal DNA in blood buffy coat specimens. The absorbance values obtained are plotted against each of the six patient categories. The horizontal bar at an absorbance of 0.5 represents the minimum cutoff value for the designation of a positive result.

use of parenteral antibiotic treatment prior to hospital admission. Lumbar puncture is being undertaken less frequently. A modest trend away from the use of lumbar puncture was noted in a recent audit of pediatric patients with meningococcal disease in west Gloucestershire (4a). Confirmation of the diagnosis is extremely important not only in the treatment of the patient but also in the management and counseling of contacts. Making a secure diagnosis is even more important when clusters of suspected cases occur, e.g., in a school or university. A PCR could be developed to be able to distinguish serogroups, serotypes, and serosubtypes. It should also be possible to extend the test described here to tissue aspirates and other clinical specimens and to the detection of other organisms.

Accurate case ascertainment will be vital to assess the need for meningococcal vaccines and to measure their efficacies. In the present study, PCR confirmed the diagnosis in 15 patients with meningococcal disease from whom there were no other currently accepted criteria for laboratory confirmation (patient categories 3 and 4). This increased the number of confirmed cases of meningococcal disease in our selected patient group by 60%. The rapid increase in the use of preadmission parenteral antibiotic treatment in patients suspected of having meningococcal disease means that a diagnosis will not be confirmed by a positive culture result for an increasing proportion of patients. Without PCR, a substantial and increasing number of cases of meningococcal disease will remain undiagnosed. The development of a specific and sensitive PCR test with peripheral blood as a substrate represents a significant and timely advance in the diagnosis of meningococcal disease.

ACKNOWLEDGMENTS

This work was supported by the National Meningitis Trust. Work involving use of the ABI373A was supported by The Wellcome Trust.

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